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Detection of *Borrelia burgdorferi* Nucleic Acids after Antibiotic Treatment Does Not Confirm Viability

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The persistence of dormant, noncultivable *Borrelia burgdorferi* after ceftriaxone treatment was examined. *B. burgdorferi* isolates were cultivated in Barbour-Stoenner-Kelly medium in the presence or absence of ceftriaxone, and cultures were monitored for up to 56 days. Viability of *B. burgdorferi* was assessed by subculture, growth, morphology, and pH (as a surrogate for metabolic activity). In addition, the presence of *B. burgdorferi* DNA and mRNA was assayed by PCR and by real-time reverse transcription (RT)-PCR, respectively. Spirochetes could not be successfully subcultured by day 3 after exposure to ceftriaxone. In cultures treated with ceftriaxone, the pH of the culture medium did not change through day 56, whereas it declined by at least 1 pH unit by 14 days in untreated cultures. These results suggest that *B. burgdorferi* viability is rapidly eliminated after antibiotic treatment. Nevertheless, DNA was detected by *B. burgdorferi*-specific PCR for up to 56 days in aliquots from both ceftriaxone-treated and untreated cultures. In addition, although ceftriaxone treatment resulted in a reduction in the quantities of transcript for *ospC*, *ospA*, *flaB*, and *pfk*, certain mRNAs could be detected through day 14. Transcript for all 4 genes was essentially undetectable after 28 days of treatment. Taken together, the results suggest that *B. burgdorferi* DNA and mRNA can be detected in samples long after spirochetes are no longer viable as assessed by classic microbiological parameters. PCR positivity in the absence of culture positivity following antibiotic treatment in animal and human studies should be interpreted with caution.

Lyme disease is caused by *Borrelia burgdorferi*, the most commonly reported vector-borne infection in North America with approximately 33,000 confirmed and probable cases in 2011 (1). Objective manifestations of this infection, such as the skin lesion erythema migrans, meningitis, carditis, or arthritis, typically respond to antibiotic therapy (2). Subjective symptoms such as fatigue and arthralgias may persist despite antibiotic therapy in approximately 10 to 15% of patients treated for erythema migrans (3, 4). Whether the frequency of such symptoms exceeds background rates in the general population, however, remains unproven (3). Nevertheless, the existence of patients with posttreatment symptoms has led to several randomized placebo-controlled trials of retreatment with antibiotic therapy along with intensive investigations of such patients by culture and PCR to find evidence of persistence of *B. burgdorferi* (5–7). In U.S. patients, no evidence has been provided to date for long-term persistence of *B. burgdorferi* in blood, cerebrospinal fluid, skin, or synovial fluid specimens after antibiotic treatment (8, 9). In addition, symptom relief was not achieved by retreatment in the controlled trials.

The question of whether there might be residual infection in treated patients has also prompted a number of studies of the efficacy of antibiotic therapy in eradicating *B. burgdorferi* from infected animals (10–12). These studies have had various results, and a number of methodological limitations have made the findings difficult to interpret (12, 13). Some of these investigations have concluded that viable *B. burgdorferi* persists in treated animals but in a dormant, noncultivable state (11, 14). Analogies have been made with the well-established phenomenon of persistence of small subpopulations in studies of other bacteria when exposed to cidal antibiotics *in vitro* (15, 16). The strongest biochemical evidence of persistence of viable *B. burgdorferi* cells has been the demonstration of mRNA of *B. burgdorferi* in some of the treated animals, based on the assumption that mRNA would be present only if the borrelial cells were alive.

In this study, the persistence of dormant but noncultivable cells of *B. burgdorferi* after exposure to ceftriaxone *in vitro* was examined by several measures of viability, including the presence of mRNA.

MATERIALS AND METHODS

***Borrelia burgdorferi* strains, culture conditions, and experimental design.** *B. burgdorferi* isolates B31A3 (17) and BL206 (18) were grown at 34°C to a density of 1×10^8 cells/ml in Barbour-Stoenner-Kelly (BSK) medium (Sigma) supplemented with 6% rabbit serum (Sigma). The cultures were diluted to 1×10^5 cells/ml in 700 ml of fresh BSK medium and cultured further for 3 days at 34°C, at which time they had reached a density of 1×10^7 cells/ml. The culture was equally divided into two flasks, and ceftriaxone (Sigma) was added to one flask to a final concentration of 15 μg/ml. The two 350-ml culture flasks (with or without ceftriaxone) were each split into 7 tubes containing 50-ml portions of the respective cultures, and incubation was continued at 34°C. One 50-ml tube of a ceftriaxone-treated culture and one 50-ml tube of an untreated culture were removed on days 0, 1, 3, 7, 14, 28, and 56, from which aliquots were obtained to perform assays for bacterial viability and for detection of spirochetal nucleic acids, as described below. Sampling from individual tubes was done at only a single time point, based on the duration of incubation (e.g., the tubes used for assays for the day zero time point were not used again for assays performed on any other day).

Bacterial growth. Ten-microliter aliquots were removed from the 50-ml tubes at the indicated times, and spirochetes were enumerated both by dark-field microscopy and by staining with acridine orange as previously described (19).

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TABLE 1 Primers used in this study

Protein (gene)	Forward primer (coordinates)[a]	Reverse primer (coordinates)[a]	Use
Enolase (*bb0337*)	AAGGGCATTCTAAGTGGCAAAGGG (345633–345656)	ACGCTCTTACCGTGGTATTCAGCA (346339–346316)	RT-PCR
Pyruvate kinase (*bb0348*)	CCTGCAGAAGATGTACCCATTGCT (357742–357765)	TGGAACACTTGCTCTGTAGGTTGC (358176–358153)	RT-PCR
Phosphofructokinase (*bb0727*)	AAGTTATGGGACGGGATTCTGGCT (766124–766147)	TTTTGTCTTACCAGCCATTGCA (766572- 766551)	RT-PCR
Phosphofructokinase (*bb0727*)	GCTAATGCCAGTGATTCGCTTT (766495–766516)	TTTTGTCTTACCAGCCATTGCA (766572- 766551)	Real-time RT-PCR
Outer surface protein A (*bba15*)	GGGAATAGGTCTAATATTAGCC (9410–9432)	TTTCAACTGCTGACCCCTC (10180–10160)	PCR, RT-PCR
Outer surface protein A (*bba15*)	TGAAGGCGTAAAAGCTGACAAA (9684–9705)	TTCTGTTGATGACTTGTCTTTGGAA (9825–9801)	Real-time RT-PCR
Outer surface protein C (*bbb19*)	GGGAAAGATGGGAATACATCTGC (16971–16993)	CTGCCACAACAGGGCTTGTAAGC (17515–17493)	RT-PCR
Outer surface protein C (*bbb19*)	CAGGGAAAGATGGGAATACATCTGC (16967–16991)	CGCTTCAACCTCTTTCACAGCAAG (17095–17071)	Real-time RT-PCR
Flagellin, *flaB* (*bb0147*)	GCAGCTAATGTTGCAAATCTTTTC (147998–148015)	TGAGCTCCTTCCTGTTGA (148089–148066)	Real-time RT-PCR

[a] Coordinates are based on the position in the genome sequence of strain B31.

Subculture. Aliquots (0.5 ml) were removed from the 50-ml tubes at the indicated times, and cells were harvested by centrifugation at 8,000 × *g* for 10 min. For each aliquot, cell pellets were washed twice with 5 ml sterile phosphate-buffered saline (PBS) to remove residual antibiotic and resuspended in 200 μl of fresh BSK medium without antibiotic. A 100-μl volume of this cell suspension was inoculated into 5 ml fresh BSK medium. Cultures were monitored for growth by dark-field microscopy for up to 4 weeks as previously described (19).

Subculture assay sensitivity for both strain B31A3 and strain BL206 was assessed by limiting dilution using a dilution series ranging from 0.1 to 1,000 spirochetes. Organisms were inoculated in triplicate into 4.0 ml of BSK medium and grown at 34°C. Cultures achieved a density of 1×10^6 cells/ml in 3 to 14 days, depending on the initial inoculum. With an initial inoculum of 10 cells, all 3 replicate cultures were positive by dark-field microscopy. At an initial inoculum of one cell, two of three replicates were positive. Cultures remained negative when the initial inoculum was 0.1 organism.

Bacterial viability. Spirochete viability was assessed by means of the BacLight bacterial viability kit (Molecular Probes, Eugene, OR), according to the manufacturer's protocol. A 10-μl aliquot was removed, and viable (green) and dead (red) organisms were quantified by fluorescence microscopy from a minimum of eight fields selected at random. The percentage of viable cells was calculated as the number of green fluorescent bacteria (live) in the aliquot divided by the total number of cells in the aliquot (red + green) multiplied by 100.

DNA extraction and PCR. On the appropriate day, DNA was isolated from a 1.0-ml aliquot from the 50-ml tubes using the Gentra Puregene Cell kit (Qiagen) as per the manufacturer's instruction and resuspended in 50 μl of DNase-free water. The amount of DNA in 5 μl was used for PCR amplification to detect the presence of the outer surface protein A gene (*ospA*). Each PCR mixture, in a total volume of 30 μl, contained 10 ng of DNA, deoxynucleoside triphosphate (dNTP) (250 μM), 10 pmol of gene-specific primers (Table 1), and 1.25 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN) in PCR buffer (Roche) containing 15 mM $MgCl_2$. Thirty-five amplification cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s were carried out, and the amplified product was analyzed on a 1% agarose gel and visualized by ethidium bromide staining. Multiple control reaction mixtures lacking DNA were included in all PCR runs. The sensitivity for this PCR assay was 10 organisms.

RNA isolation and reverse transcription (RT)-PCR. On the appropriate day, total RNA was extracted from up to 5×10^8 cells (or 40 ml obtained from the 50-ml tubes for ceftriaxone-treated cells) using the Totally RNA isolation kit (Ambion) as per the manufacturer's protocol. All RNA samples were treated twice with DNase by means of the Turbo DNA free kit (Ambion) in order to remove any contaminating genomic DNA. RNA quality was assessed by gel electrophoresis, and concentrations were measured spectrophotometrically using an Eppendorf Biophotometer. $A_{260/280}$ ratios were 1.7 to 2.2.

First-strand cDNA was synthesized by reverse transcription of 2 μg of total RNA in a 20-μl reaction volume containing 10 units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI), 500 ng of random hexamers (Promega), 250 μM dNTP, and RNasin (5 U) in AMV buffer (Promega). The reaction mixture was incubated at 42°C for 60 min. Reverse transcriptase was inactivated by heating at 95°C for 5 min, and the resultant cDNA was stored at −20°C until further use. A control reaction mixture lacking reverse transcriptase was carried out for each primer set using total RNA to ensure that no contaminating DNA was present.

PCR was performed with gene-specific primers (Table 1) for enolase, pyruvate kinase, phosphofructokinase, OspA, and OspC. Each PCR mixture contained 40 ng of cDNA, 250 μM dNTP, 10 ng of each primer, and 1.25 U of *Taq* DNA polymerase (Roche Molecular Biochemicals) in 10 mM Tris-HCl–1.5 mM $MgCl_2$–50 mM KCl, pH 8.3. The PCR conditions were 95°C for 2 min followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min. The PCR products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining. Multiple control reaction mixtures lacking DNA were included in all PCR runs.

Quantitative real-time RT-PCR. For each reaction, 4 ng of cDNA (generated as described above) and 20 pmol of gene-specific primers (*flaB*, *ospA*, *ospC*, and *pfk* [Table 1]) were used, and PCR amplification was performed in a 25-μl reaction mixture containing 1× SYBR green PCR master mix (Roche Diagnostics). Oligonucleotide primers for real-time PCR were designed using Primer Express software, version 2.0 (Applied Biosystems, CA). All reactions were carried out on the ABI Prism 7900 HT SDS system using thermal cycling parameters consisting of 2 min at 55°C and denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCRs were performed in duplicate for each RNA sample. To verify the purity of the PCR product, melting curve analyses were performed. In addition, a no-template reaction control was included for each primer set.

DNA samples containing 10 to 10,000 copies of genomic DNA were included in each real-time RT-PCR experiment for generation of a stan-

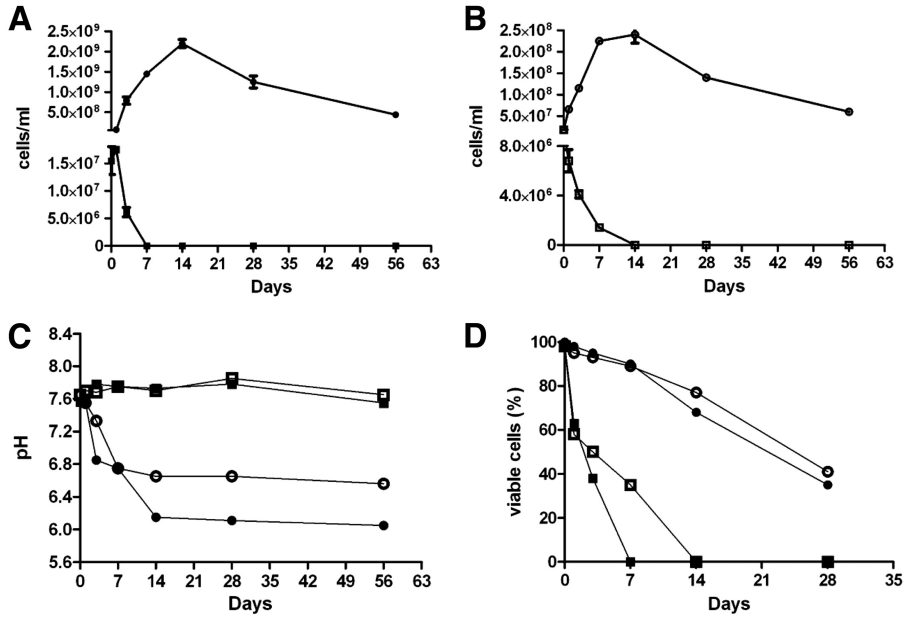

FIG 1 Survival of *B. burgdorferi* after ceftriaxone treatment. (A and B) Growth of *B. burgdorferi* B31A3 (A) or BL206 (B) in BSK medium. Values are means ± standard errors of the means (SEM) for measurements from duplicate cultures. (C) Metabolic activity of *B. burgdorferi* in BSK medium (based on acidification of medium). (D) Viable *B. burgdorferi* cells as assessed by fluorescence microscopy. ●, B31A3; ■, B31A3 plus ceftriaxone; ○, BL206; □, BL206 plus ceftriaxone.

dard curve. A plot of cycle threshold (C_T) value versus log amount of known standard DNA was generated. The quantity of template cDNA was calculated from the C_T values obtained using Applied Biosystems SDS 2.1 software as described previously (20). All standard curves conformed to a linear relationship ($r^2 > 0.98$) and had an amplification efficiency of 95 to 100%. Gene expression measurements were normalized to μg of total RNA (i.e., the transcript abundance of each gene was calculated per μg of RNA). Changes in transcript levels for each gene were calculated relative to day zero values, which were set to 100.

Statistics. The statistical significance of observed differences in pH and viability between antibiotic-treated and untreated cultures was evaluated by a paired Student *t* test with two-tailed *P* values and 95% confidence interval using GraphPad PRISM software (V 5.0; La Jolla, CA).

RESULTS

***B. burgdorferi* growth.** *B. burgdorferi* strains B31A3 and BL206 were grown in the absence or presence of 15 μg/ml ceftriaxone at 34°C for 56 days, and cell density was monitored at the indicated time intervals by dark-field microscopy. Growth profiles are presented in Fig. 1A and B. In the absence of antibiotic, both isolates grew exponentially until day 14, reaching cell densities of 2.3×10^9 (B31A3) and 2.3×10^8 (BL206). This was followed by a gradual decrease in cell density over the subsequent 6 weeks of cultivation; by day 56, cell densities for B31A3 and BL206 were 4.4×10^8 and 6×10^7, respectively. In contrast, bacterial growth was arrested in antibiotic-treated cultures and the cells began to disintegrate by day 3; at the next sampling time on day 7, cells could not be enumerated by microscopy.

***B. burgdorferi* metabolic activity.** As a measure of metabolic activity, the pHs of the media were monitored during growth (Fig. 1C). The pH of the untreated cultures declined gradually from 7.6 to 6.7 (BL206) or 6.2 (B31A3) by day 14, indicating that cells were actively metabolizing the nutrients in the medium and producing acid. No further decrease in pH was observed beyond this time. In contrast, the pH remained essentially unchanged (7.5 to 7.8) in ceftriaxone-treated cultures throughout the duration of the experiment. The observed change in pH between antibiotic-treated and untreated cultures was statistically significant ($P = 0.011$ for both B31A3 and BL206).

Spirochetal motility, morphology, and viability. By the third day of antibiotic treatment, spirochetes were nonmotile and only 50% retained typical spirochete morphology as observed by either dark-field or fluorescence microscopy (not shown). In the absence of antibiotic treatment, cells were motile and morphologically intact through day 14, after which they also became nonmotile and began to disintegrate. Figure 1D shows the results of "live/dead" staining to assess the viability of *B. burgdorferi*. At day 7, approximately 90% of untreated *B. burgdorferi* cells remained viable (i.e., stained green), whereas 0% (B31A3) or 37% (BL206) of treated spirochetes were viable based on this measure (Fig. 1D). It should be noted that in the ceftriaxone-treated cultures the majority of the spirochetes had disintegrated after day 7. It was therefore not possible to perform viability staining, as the cells were all fragmented beyond this time point. The reduction in viability between treated and untreated spirochetes as measured by this assay was statistically significant (B31A3, $P = 0.013$; BL206, $P = 0.0094$).

To assess viability, cell aliquots were removed from the cultures, washed to remove residual antibiotic, and subcultured in fresh medium. Spirochetes removed from the primary cultures 24 h after antibiotic addition could be successfully subcultured from either treated or untreated cultures (Table 2). Whereas subculture was successful with aliquots from untreated cultures through day 14, *B. burgdorferi* could not be recovered from ceftriaxone-treated cultures at the next time point that subcultures were attempted on day 3 and thereafter.

Detection of *B. burgdorferi* DNA and mRNA. The results presented above indicate that spirochetes are no longer cultivable at 3 days after ceftriaxone treatment. It has been suggested that detec-

TABLE 2 Detection of *B. burgdorferi* after ceftriaxone treatment by culture or PCR[a]

No. of days after ceftriaxone addition	Subculture		PCR	
	−Ctx	+Ctx	−Ctx	+Ctx
0	+	+	+	+
1	+	+	+	+
3	+	−	+	+
7	+	−	+	+
14	+	−	+	+
28	−	−	+	+
56	−	−	+	+

[a] Strain B31A3 data are shown; identical results were obtained with BL206. PCR assays were for detection of *ospA*. Ctx, ceftriaxone.

tion of *B. burgdorferi* DNA by PCR amplification may serve as a surrogate for culture in assessing viability (21, 22). Aliquots of culture were removed at various time points and assessed for the presence of *ospA* DNA by PCR. Despite the fact that *B. burgdorferi* cultivability was abolished by the third day after ceftriaxone treatment, *ospA* DNA could be detected in culture aliquots throughout the duration of the experiment, i.e., up to 56 days (Table 2). This indicates that *B. burgdorferi* DNA persists long after culture evidence of live organisms could be demonstrated.

RNA is less stable than DNA; the average mRNA half-life in most bacterial species is approximately 3 min and typically does not exceed 1 h even for unusually stable mRNA species (23, 24). It has therefore been assumed that detection of mRNA implies metabolic activity, as the lability of RNA would result in its degradation very shortly after cell death (25). Thus, the presence of transcript for five genes was explored by RT-PCR in ceftriaxone-treated *B. burgdorferi*. These included those for *ospA* and *ospC*, whose transcripts are abundantly expressed in *B. burgdorferi* cultured in BSK at 37°C, as well as three mRNAs for genes encoding enzymes of the glycolytic pathway: enolase, pyruvate kinase, and phosphofructokinase. Results are summarized in Table 3. The ability to detect mRNA varied with the specific transcript. In untreated cultures, mRNA for all targets (except *eno*) was detectable for up to 4 weeks; for *eno*, transcript was not observed after day 7. In ceftriaxone-treated cultures, transcripts for *ospA*, *ospC*, and *eno* were not detected after day 3; however, *pyk* and *pfk* mRNA persisted for up to 7 days.

Quantitation of *B. burgdorferi* transcripts by real-time RT-PCR. Although the RT-PCR results described above suggested that transcript can persist long after cell cultivability has been abrogated, it is possible that mRNA is mostly degraded after a short period of time. To assess this possibility, real-time RT-PCR was performed in order to quantitate the transcript levels for several genes (Table 4). In untreated cultures, transcript levels for *flaB*, *ospA*, and *ospC* increased between 4- and >100-fold through day 7 and then declined gradually by day 28 to <20% of the original transcript levels. *pfk* mRNA remained essentially unchanged through day 14; levels were reduced to 9% by day 28. Ceftriaxone treatment resulted in an immediate decline in *ospA* and *ospC* mRNA; transcript levels were <5% of the original levels after 3 days. *flaB* mRNA levels diminished to 17% within 24 h of treatment but remained at that level through 14 days. *pfk* transcript levels followed a similar pattern but were diminished by only one-half. Transcript for all 4 genes was essentially undetectable after 28 days of treatment. Taken together, the RT-PCR findings demonstrate that bacterial mRNA can be detected for substantial time periods beyond abrogation of viability as measured by several microbiological criteria.

DISCUSSION

Numerous methods have been employed to assess bacterial viability. Classically, cultivation in either solid or liquid media has been considered the gold standard. Since many bacteria are not cultivable, surrogate methods such as measurement of metabolic activity, membrane integrity, or presence of bacterial nucleic acids have been employed (25, 26). In this study, we have utilized and compared all of these approaches to assess the viability of *B. burgdorferi* after ceftriaxone exposure. Measurements based on cultivation, metabolic activity, or cellular integrity indicated that the bacteria rapidly lose viability following exposure to the antibiotic.

The existence of antibiotic-tolerant persister cells after antimicrobial treatment has been described for many bacterial species. These "persister" cells are not antibiotic-resistant mutants; rather, they are phenotypic variants that arise spontaneously in an otherwise genetically identical cell population (15, 16, 27). Persisters can be subcultured, and recultivation of the persisters in the same antibiotic results in bacterial killing with a pattern that is essentially identical to that of the original cell population (i.e., virtually all cells are antibiotic sensitive, with a small proportion again becoming persisters). The results presented here clearly show that unlike the experience with certain other bacteria, persister cells are not found after exposure of *B. burgdorferi* to ceftriaxone *in vitro*. Furthermore, our findings do not support the hypothesis that exposure to ceftriaxone will induce viable *B. burgdorferi* to alter its phenotype to become transiently or permanently noncultivable,

TABLE 3 Detection of *B. burgdorferi* mRNA after ceftriaxone treatment[a]

No. of days after Ctx addition	*ospA*		*ospC*		Enolase gene		Pyruvate kinase gene		Phosphofructokinase gene	
	−Ctx	+Ctx	−Ctx	+Ctx	−Ctx	+Ctx	−Ctx	+Ctx	−Ctx	+Ctx
0	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
7	+	−	+	−	+	−	+	+	+	+
14	+	−	+	−	−	−	+	−	+	−
28	+	−	+	−	−	−	+	−	+	−
56	−	−	−	−	−	−	−	−	−	−

[a] Strain BL206 data are shown; identical results were obtained with B31A3; Ctx, ceftriaxone.

TABLE 4 Relative levels of selected transcripts after ceftriaxone treatment[a]

No. of days after Ctx addition	*flaB*		*ospA*		*ospC*		Phosphofructokinase gene	
	−Ctx	+Ctx	−Ctx	+Ctx	−Ctx	+Ctx	−Ctx	+Ctx
0	100	100	100	100	100	100	100	100
1	183	17	232	1.6	49	7.3	77	72
3	352	21	1417	4.6	13257	0.6	76	49
7	415	17	238	3.0	586	0.2	313	41
14	43	19	19	1.5	597	0.3	62	53
28	20	0.4	1.5	0	4.6	0	9.0	0

[a] Strain BL206 data are shown; identical results were obtained with B31A3; transcript levels are relative to the level prior to ceftriaxone addition, which was set at 100. Ctx, ceftriaxone.

such as by losing key genetic elements, "encysting," or changing morphology to round bodies (11, 14, 28, 29).

We used a ceftriaxone concentration of 15 μg/ml to replicate the minimum drug level achieved in serum when the standard dose of 2 g intravenously (i.v.) daily is prescribed to treat Lyme disease. We did not determine the MIC of the borrelial strains utilized in this study to ceftriaxone, but in other studies with the same strains the MIC values ranged from 0.025 to 12 μg/ml (14, 30–32).

None of the assessed molecular parameters of cell viability, including detection of mRNA, accurately predicted the time of cell death, as assessed by subculture or spirochetal morphology. In addition, previous studies have emphasized the importance of completely eliminating DNA from RNA preparations in order to avoid false-positive results (33). In the current study, some mRNAs could be detected by real-time RT-PCR for up to 14 days, whereas others disappeared within 3 days after ceftriaxone exposure. Such variability in mRNA persistence has been described by others (33). mRNA might persist for longer or shorter periods *in vivo*. Based on experience with other microorganisms, persistence of borrelial mRNA is likely to be dependent on the cause of cell death, on the size, region, and type of the mRNA being targeted, on the level and activity of the ribonucleases in *B. burgdorferi* cells, on the physiological state of the bacterial cell population, and on the extracellular environmental conditions (34–36). mRNA might, in theory, remain intact for relatively long periods of time if cells are destroyed by treatments that inactivate RNase but not the mRNA (37). For *B. burgdorferi* in particular, persistence of mRNA may also depend on whether the dead spirochetal cell or some fraction of a cell becomes enmeshed in a host-derived fibrinous or collagenous matrix, a hypothetical possibility that seems to explain many of the unusual features of Lyme arthritis (9, 10).

It is unclear whether our findings can be extrapolated to *B. burgdorferi* infections *in vivo*. It has been suggested that *B. burgdorferi* may be sequestered in protective niches during animal infection such that antibiotics might be less effective (11, 12, 41). On the other hand, it is also possible that the host's immunologic response and/or other inhibitory factors found *in vivo* could result in a more rapid decrease in viability after exposure to an antibiotic than occurs after the same level of antibiotic exposure during *in vitro* cultivation. An advantage of the *in vitro* approach is that it provides the capability to ensure that the concentration of antibiotic is similar to that found in patients receiving the drug. Indeed, in only one of the reported animal studies on the treatment of *B. burgdorferi* infection was the nadir concentration of ceftriaxone likely to have been as high as that achieved in humans (32). In spite of the acknowledged limitations of an *in vitro* study, the results presented here serve as a proof of principle that detection of spirochetal nucleic acids does not necessarily correlate with the presence of intact, metabolically active organisms. Thus, a positive PCR or RT-PCR result, in the absence of a positive culture, must be interpreted with caution.

Investigators have used various endpoints in treatment studies of animals infected with *B. burgdorferi*. Some have relied on cultivability of the spirochete as the sole measure of antibiotic efficacy, as is conventionally employed for other microorganisms (32, 38, 39), whereas others have required complete elimination of all spirochetal nucleic acids and proteins (11, 14, 22, 28, 40, 41). Our study and those of others suggest that the antimicrobial activity of ceftriaxone can be reliably assessed based on cultivability and that cultivation is a more accurate reflection of cell viability. Other endpoints that focus on persistence of spirochetal components such as DNA and mRNA, although of less direct importance to the issue of antibiotic efficacy, might be pertinent in explaining the persistence of inflammation in patients with antibiotic-refractory Lyme arthritis, an uncommon inflammatory condition in joints that continues after apparent eradication of viable *B. burgdorferi* (9, 10). The persistence of spirochetal components in the absence of inflammation (12), however, would seem to be much less biologically relevant and would be highly unlikely to explain nonspecific clinical symptoms such as fatigue.

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